chemical function of pseudouridine. Both of its NH groups are capable of hydrogen bonding with adenosine, but it remains an open question if their biochemical function in tRNA is the same as observed in the present study.

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Subunit Structure of Glucose Oxidase from *Aspergillus niger*†

James J. O'Malley* and Judy L. Weaver

ABSTRACT: Glucose oxidase from *Aspergillus niger* is a branched protein containing 16% carbohydrate and two flavin-adenine dinucleotide cofactors per molecule. The properties of the native enzyme have been well studied, but there is relatively little known about the denatured enzyme. In this study we examined the structural properties of the denatured enzyme, using the techniques of light scattering, sedimentation velocity, intrinsic viscosity, and amperometric titration to determine whether glucose oxidase is a single or multichain (subunit) enzyme. The results of these studies indicate that glucose oxidase is a subunit enzyme. The native enzyme has a molecular weight of 160,000 and light-scattering measurements in 6 M guanidine hydrochloride indicate the denatured enzyme has the same molecular weight as the native enzyme. However, chemical reduction with β-mercaptoethanol of the enzyme's two disulfide bonds results in the formation of molecular species with molecular weights of 80,000. Schlieren patterns taken during sedimentation velocity runs show single sharp sedimentation peaks for both the denatured and the denatured-reduced species. These data are consistent with a model for glucose oxidase in which the enzyme is composed of two polypeptide chains, equal in molecular size, which are covalently linked by disulfide bonds.

Glucose oxidase is a flavin containing glycoprotein which catalyzes the oxidation of glucose to gluconic acid (Müller, 1928). The enzyme isolated from *Aspergillus niger* contains approximately 16 wt % carbohydrate and these sugars are thought to be present as oligomeric polysaccharides covalently attached to the polypeptide chain *via* serine, glutamic acid, and aspartic acid residues (Pazur *et al.*, 1963, 1965). The native enzyme is also known (Franke and Deffner, 1939; Pazur and Kleppe, 1964) to contain two molecules of flavin-adenine dinucleotide (FAD).[1] These flavin cofactors are responsible for the oxidation–reduction properties of the enzyme and available evidence suggests they are firmly bound, but not covalently linked, to the polypeptide portion of the enzyme. Denaturation generally results in flavin release from the enzyme and, concomitantly, changes in the absorption and fluorescence emission spectra of the flavin are observed (Swoboda and Massey, 1966).

Many measurements of the molecular weight of the native enzyme have been made and, while the reported values range from 150,000 (Pazur and Kleppe, 1964) to 186,000 (Swoboda and Massey, 1965), most of the data fall in the range of 155,000 ± 5000. In view of the interest in glucose oxidase, it is surprising that not until recently (O'Malley and Weaver, 1971) was the molecular weight of the denatured enzyme measured and the possibility of subunit structure seriously considered. The present study, using glucose oxidase isolated from *A. niger* and techniques such as light scattering, sedimentation velocity, and intrinsic viscosity, points to a subunit structure for this enzyme. The data are consistent with a model in which the native enzyme is composed of two polypeptide chains equal in molecular size and covalently linked by disulfide linkages.

Experimental Section

Materials. Glucose oxidase (EC 1.1.3.4) from *A. niger* was obtained from Worthington Biochemicals as a highly purified, salt-free lyophilized powder with a specific activity of 136 IU/mg. The following materials were purchased from sources as indicated: Gdn·HCl and urea from Mann; guanidine thiocyanate from K & K; dithiothreitol from Pierce;

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[1] Abbreviations used are: FAD, flavin-adenine dinucleotide; Gdn·HCl, guanidine hydrochloride; βME, β-mercaptoethanol.

iodoacetamide from Aldrich; βME and phenylmercuric acetate from Eastman; sodium sulfite, sodium acetate, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate from Baker.

Samples of reduced and alkylated glucose oxidase were prepared by reducing the enzyme with 0.1 M βME in 6 M Gdn·HCl at pH 8.0 for 1 hr and then alkylating with 0.12 M iodoacetamide for 1 hr at pH 8.0. The reaction solution was protected from light during this procedure and the enzyme was recovered by extensive dialysis against water and lyophilization. Yields were generally between 80 and 90% and spectral analysis confirmed that the flavin–adenine dinucleotide cofactors were removed by this procedure.

Amperometric Titrations. An experimental arrangement similar to that for conventional polarography was used to determine the number of sulfhydryl and disulfide groups in glucose oxidase. Phenylmercuric acetate was used to titrate the thiol groups. The titration setup consisted of a small cell fitted with a nitrogen bubbler, a dropping mercury electrode, a saturated KCl electrode and a micro syringe for adding phenylmercuric acetate.

The solutions were carefully deoxygenated before beginning each experiment and nitrogen was passed through the solution after each addition of titrant. The dropping mercury electrode was polarized at a potential where only the phenylmercuric acetate was reduced and the change in current with addition of phenylmercuric acetate was monitored on a Beckman Electroscan 30.

Intrinsic Viscosity. Measurements were made in a No. 75 Cannon-Ubbelohde semimicro dilution viscometer at 25° and efflux times for water and aqueous guanidine hydrochloride solutions were in excess of 120 sec. A working volume of 2.0 ml was generally used for the highest solute concentration and subsequent dilutions were made by adding solvent directly into the viscometer. In this manner, the viscosity at several concentrations could be measured without removing the viscometer from the thermostatted bath.

The intrinsic viscosity (η) values were obtained by extrapolating the reduced viscosity *vs.* concentration plots to $c = 0$ and the data are expressed in milliliters per gram.

$$\eta_{red} = \frac{1}{c}\frac{\eta - \eta_0}{\eta_0} = (\eta) + K(\eta)^2 c \qquad (1)$$

The time dependence of the viscosity was checked in each experiment to ensure that conformational equilibrium was established. This was usually done by monitoring the viscosity of the most concentrated protein solution as a function of time and, on several occasions, the most dilute solution at the end of the experiment was also monitored. In most cases, the viscosity of the protein solutions did not change after 1 hr at 25°. With solutions containing sodium sulfite it was necessary to keep them under nitrogen in order to obtain stable readings.

Light Scattering. A Sofica light-scattering photometer thermostatted at 25° was used for the light-scattering measurements. Unpolarized filtered light with a wavelength of 546 nm was used as the incident radiation and the scattering intensity was monitored at angles ranging from 30 to 150°.

The light-scattering results are presented in terms of the quantity Kc/R_θ. The concentration, c, is given in grams per milliliter, R_θ is the reduced intensity of scattering (corrected for solvent contribution) at angle θ to the incident beam and K is equal to a series of constants for a given solvent–solute system. The final form of the light-scattering equation is shown as

$$\frac{Kc}{R_\theta} = \frac{2\pi^2 n_B^2 (\partial n/\partial c)^2}{\lambda^4 N R_B}\frac{1 + \cos^2\theta}{\sin\theta}\frac{cI_{B,90}}{\Delta I_\theta} \qquad (2)$$

The parameters in eq 2 are as follows: n_B is the refractive index of the calibrating liquid benzene, λ is the wavelength *in vacuo*, N is Avogadro's number, R_B is the Rayleigh ratio of benzene (1.58×10^{-5} cm^{-1}, Coumou, 1960), $\partial n/\partial c$ is the refractive index increment, $1 + \cos^2\theta$ is a correction factor for unpolarized light, $\sin\theta$ is a correction factor for the change in scattering volume with angle, $I_{B,90}$ is the light scattered by benzene at 90°, and ΔI_θ is the excess scattering of the solution over the solvent at the angle θ.

Protein solutions were freed of dust by filtering the solutions through 0.20 μ pore size Millipore filters directly into the precleaned cylindrical scattering cells. The cells were tightly covered and the exact concentrations were determined by spectrophotometric analysis after completing the light scattering experiment.

Refractive index increments were measured with a Brice-Phoenix differential refractometer at 25°. Light with a filtered wavelength of 546 nm was used. Five protein solutions with concentrations ranging from 2 to 10 mg per ml were employed in determining the refractive index increments. Values of 0.118 and 0.122 ml per g were found for solutions of 6 M Gdn·HCl and 6 M Gdn·HCl–0.1 M βME, respectively.

Sedimentation Velocity. A Spinco Model E analytical ultracentrifuge equipped with schlieren optics was used and all runs were made at 5° and a rotor speed of 60,000 rpm. Sedimentation coefficients were corrected to the reference state of water at 20° and 6 M Gdn·HCl at 25° according to

$$s^0_{20,w} = \frac{s_{5,NaAc}\eta_{5,NaAc}(1 - \bar{\nu}\rho)_{20,w}}{\eta_{20,w}(1 - \bar{\nu}\rho)_{5,NaAc}} \qquad (3)$$

$$s^0_{25,Gdn\cdot HCl} = \frac{s_{5,Gdn\cdot HCl}\eta_{5,Gdn\cdot HCl}(1 - \bar{\nu}\rho)_{25,Gdn\cdot HCl}}{\eta_{25,Gdn\cdot HCl}(1 - \bar{\nu}\rho)_{5,Gdn\cdot HCl}} \qquad (4)$$

where s^0 is the corrected sedimentation coefficient, s is the sedimentation coefficient determined experimentally at 5°, η is the viscosity, ρ is the density, and $\bar{\nu}$ is the partial specific volume. The viscosity and density corrections were made using literature data (Kawahara and Tanford, 1966; Reithel *et al.*, 1964; International Critical Tables, 1928) and a partial specific volume of 0.714 ml/g was used for glucose oxidase in buffer (Nakamura and Fujiki, 1968). For the enzyme in 6 M Gdn·HCl, a partial specific volume of 0.704 ml/g (0.01 ml/g less than in buffer) was assumed (Hade and Tanford, 1967).

Results

Intrinsic Viscosity. The intrinsic viscosity profile of reduced glucose oxidase in Gdn·HCl is shown in Figure 1. The native enzyme in aqueous solution has an intrinsic viscosity of 4.0 ml/g and denaturing it in solutions of increasing Gdn·HCl concentration results in substantial increases in intrinsic viscosity. At low concentrations of Gdn·HCl the viscosity only increases slightly, but between 3 and 6 M Gdn·HCl there is a very rapid increase in viscosity with increasing salt concentration. The intrinsic viscosity levels off near 30 ml/g at 6 M Gdn·HCl and does not increase on going to 8 M

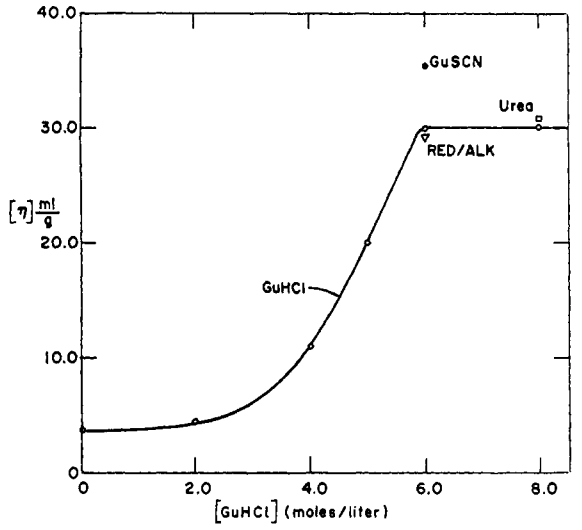

FIGURE 1: Effect of guanidine hydrochloride concentration on the intrinsic viscosity of glucose oxidase at 25°. All solutions contain 0.1 M β-mercaptoethanol as reducing agent. Data for the reduced enzyme in guanidine thiocyanate and urea are also shown. The datum point designated RED/ALK shows the intrinsic viscosity of a reduced and alkylated glucose oxidase sample after flavin removal by dialysis.

Gdn·HCl. Alkylation of the reduced enzyme to prevent oxidation does not substantially change the intrinsic viscosity of the denatured enzyme.

Intrinsic viscosity data for glucose oxidase denatured in 8 M urea and 6 M guanidine thiocyanate containing 0.1 M βME are also included in Figure 1. It appears that 8 M urea is as effective as 6 or 8 M Gdn·HCl in unfolding the enzyme and that 6 M guanidine thiocyanate is a better denaturing solvent for glucose oxidase than either concentrated solutions of urea or Gdn·HCl.

To investigate what effect flavin (FAD) release from the enzyme might have on the hydrodynamic properties, the visible absorption spectra of the native and solvent denatured enzymes were recorded (Figure 2). The spectrum of the flavin bound to the native enzyme has absorption maxima at 381 and 452 nm whereas the spectra of the solvent denatured enzyme have maxima at 377 and 448 nm. This blue shift is expected (Swoboda and Massey, 1965) on going from enzyme-bound FAD to free FAD in solution and it indicates that in 2–8 M Gdn·HCl, FAD is released from glucose oxidase and is free in solution. It appears, therefore, that flavin release does not substantially influence the viscometric properties of the enzyme since only small changes in viscosity are observed on denaturing the enzyme in solutions containing up to 3 M Gdn·HCl.

Since the intrinsic viscosity of the reduced enzyme in 6 M Gdn·HCl was substantially less than what we initially expected (see Discussion below), variables such as reducing agent concentration, pH, and temperature were evaluated while keeping the Gdn·HCl concentration constant at 6 M. In general, these parameters had little or no effect on the intrinsic viscosity. The βME concentration was varied from 0 to 0.5 M and other reducing agents such as 0.05 M dithiothreitol (Cleland, 1964) and 0.44 M sodium sulfite were tried but all the data indicated an intrinsic viscosity of 30 ± 1 ml/g. Similarly, varying the pH of the 6 M Gdn·HCl–0.1 M βME solutions between pH 2.0 and 10.5 did not produce any significant change in the intrinsic viscosity at 25°. This is not unexpected since electrostatic interactions should be of little importance in a strong electrolyte solution like 6 M Gdn·HCl. Finally, the temperature dependence of the intrinsic viscosity in 6 M Gdn·HCl–0.1 M βME was found to be small, but negative, in the 25–50° range with the values ranging from 30 ml/g at 25° to 27 ml/g at 50°. The observed behavior is typically (Flory, 1953; Tanford, 1961) found with randomly coiled polymers in thermodynamically good solvents.

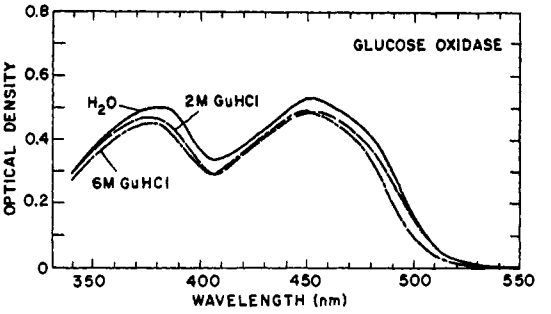

FIGURE 2: Visible absorption spectra of the flavin cofactor, FAD, in glucose oxidase before (H_2O) and after solvent (Gdn·HCl) denaturation of the enzyme.

Amperometric Titration. A series of amperometric titrations of denatured glucose oxidase in 6 M Gdn·HCl were performed to determine the sulfhydryl and disulfide content of the enzyme and to verify the reducing action of β-mercaptoethanol. Following the method of Swoboda and Massey (1965) sodium sulfite was used as the reducing agent and phenylmercuric acetate was used to titrate the available sulfhydryl groups.

The experimental results are shown in Figure 3. In titration A, 20 mg of glucose oxidase was denatured in 6 M Gdn·HCl without reducing agent and 0.023 ml of phenylmercuric acetate solution was used to titrate the available sulfhydryl groups. Based on a molecular weight of 160,000 for the enzyme, this corresponds to 1.10 sulfhydryl groups/mole of enzyme. In titration B, 20 mg of enzyme was denatured in 6 M Gdn·HCl containing 0.44 M sodium sulfite to reduce the

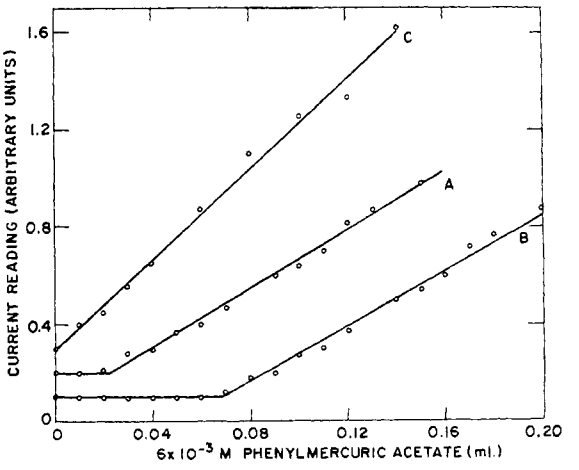

FIGURE 3: Amperometric titration of sulfhydryl groups in denatured glucose oxidase. The three titrations with phenylmercuric acetate were obtained in three separate experiments: (A) enzyme denatured in 6 M Gdn·HCl without reducing agent; (B) enzyme denatured in 6 M Gdn·HCl and reduced with 0.44 M sodium sulfite; (C) enzyme was first reduced (βME) and alkylated (iodoacetamide) in 6 M Gdn·HCl and then, after dialysis, was titrated in 6 M Gdn·HCl containing 0.44 M sodium sulfite.

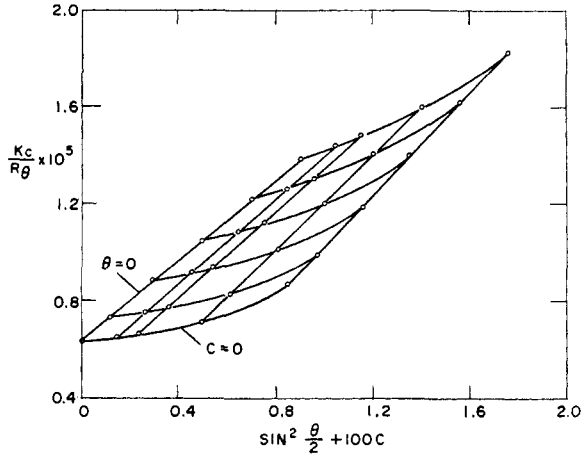

FIGURE 4: Zimm plot showing light-scattering data for glucose oxidase denatured in 6 M Gdn·HCl *without* reducing agent at 25°.

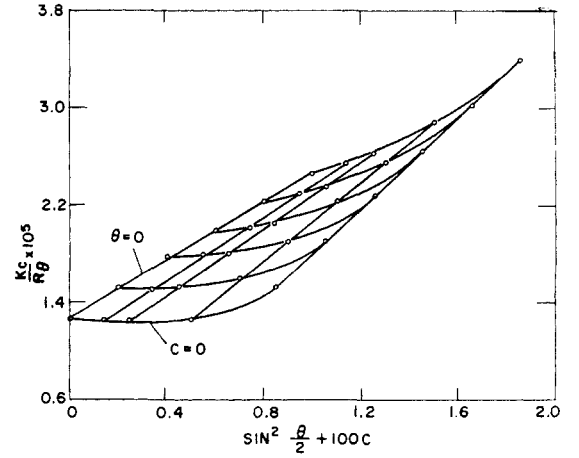

FIGURE 5: Zimm plot showing light-scattering data for glucose oxidase denatured and reduced in 6 M Gdn·HCl–0.1 M βME at 25°.

disulfide linkages. This solution required 0.068 ml of titrant which corresponds to an additional 2.16 titratable sulfhydryl groups/mole of enzyme. Since sulfite produces one sulfhydryl group per disulfide bond reduced, the above results indicate that glucose oxidase has one sulfhydryl group and two disulfide bonds per mole of enzyme. However, the lone sulfhydryl group is not available for titration when the enzyme is in its native state, and it only becomes titratable when the enzyme releases its flavin (FAD) cofactors as a result of thermal inactivation or solvent denaturation. The above results are in agreement with those reported by Swoboda and Massey (1965).

To verify that β-mercaptoethanol, which was used in most of our work, effectively reduced the disulfide bonds in the enzyme, a sample of reduced and alkylated glucose oxidase was titrated in 6 M Gdn·HCl containing 0.44 M sodium sulfite. The enzyme was previously reduced with 0.1 M βME in 6 M Gdn·HCl and its sulfhydryl groups were blocked by alkylation with iodoacetamide. Titration C in Figure 3 shows the result of this titration and it indicates that no sulfhydryls are present in the sample. Thus, the preliminary reduction with βME and alkylation with iodoacetamide reduced and blocked all the sulfite titratable sulfhydryls and disulfide groups in the enzyme.

To determine whether, on an absolute basis, all the disulfide bonds have been reduced, it is necessary to have an independent method for determining their presence in the enzyme. Using the method of Schram *et al.* (1954) which involves perchloric acid oxidation of the enzyme and analysis of the cysteic acid produced, Pazur *et al.* (1965) found two disulfides and no free sulfhydryls per mole of enzyme whereas Nakamura and Fujiki (1968) found three disulfides and one free sulfhydryl per mole of enzyme. Our results and those of Swoboda and Massey (1965) indicate the enzyme contains two disulfides and one free sulfhydryl. In view of the very small quantities of cysteine and/or cystine present in the enzyme, these independent results are in reasonable agreement and we feel that βME in 6 M Gdn·HCl effectively reduces all the disulfide bonds in glucose oxidase.

Light Scattering. Absolute molecular weight measurements of glucose oxidase in 6 M Gdn·HCl and 6 M Gdn·HCl–0.1 M βME were made by light scattering. The reduced scattering intensity was measured as a function of scattering angle and protein concentration and the data are presented in the form of Zimm plots (Zimm, 1948). An advantage of the Zimm method is that it is not necessary to assume a particular shape for the protein in order to calculate its molecular weight. The Zimm plots in Figures 4 and 5 display some curvature, but in each case the double extrapolation procedure leads to a common intercept. The intercept, $Kc/R_{\theta=0}$, is related to the molecular weight and second virial coefficient, A_2, by

$$Kc/R_{\theta=0} = M^{-1} + 2A_2c + \ldots \quad (5)$$

The data shown in Figure 4 are for the enzyme dissolved in 6 M Gdn·HCl and the intercept corresponds to a molecular weight of 160,000 g/mole. This value is within the range of molecular weights reported for the native enzyme in buffer solution and it indicates that glucose oxidase is not composed of multiple subunits associated through noncovalent interactions.

On reducing the enzyme in 6 M Gdn·HCl–0.1 M βME, a twofold decrease in molecular weight is observed. From the intercept in Figure 5, a molecular weight 80,000 g/mole is calculated. This is to be compared to the molecular weight of 160,000 g/mole found for the unreduced enzyme in 6 M Gdn·HCl. Since the mercaptan reducing agent functions by breaking intra- and intermolecular disulfide bonds in the enzyme, these results clearly indicate that glucose oxidase is composed of two polypeptide chains of approximately equal molecular weight held together by disulfide bonds.

In addition to obtaining molecular weight data from the Zimm plots, it is possible to calculate the second virial coefficient and the radius of gyration from the slopes of the $\theta = 0$ and $c = 0$ lines, respectively. For the enzyme in 6 M Gdn·HCl, an A_2 value of 4.3×10^{-4} (cm^3 mole)/g^2 was calculated, whereas a value of 6.6×10^{-4} (cm^3 mole)/g^2 for A_2 was obtained for the reduced enzyme in 6 M Gdn·HCl–0.1 M βME. These second virial coefficients are in the range expected for randomly coiled molecules and the values of $A_2M^{1/2}$ for the reduced enzyme are in agreement with the correlation of $A_2M^{1/2}$ with molecular weight reported by Lapanje and Tanford (1967) using osmotic pressure data for a number of proteins in this solvent system. This suggests a similarity among these various polypeptide chains in their thermodynamic interaction parameters. Accurate estimates of the radius of gyration could not be made because of the curvature in the $c = 0$ line.

Sedimentation Velocity. Single, sharp sedimentation peaks were observed in all of the velocity experiments. The sedimentation velocities of glucose oxidase were measured in 0.1 M sodium acetate buffer at pH 5.5 and a $s^0_{20,w}$ value of 8.0 S (10^{-13} cm/sec) was calculated from the data. This is in good agreement with data of Pazur and Kleppe (1964), Swoboda and Massey (1965), and Swoboda (1969).

An estimate of the molecular weight can be made by correlating the sedimentation coefficient with the diffusion constant and the partial specific volume (Flory, 1953) as shown in eq 6. A molecular weight of 166,000 g/mole is calculated

$$M = \frac{RTs^0_{20,w}}{(1 - \bar{v}\rho)D} \quad (6)$$

using a value of 4.12×10^{-7} cm²/sec for the diffusion constant (Swoboda and Massey, 1965) and 0.714 ml/g for the partial specific volume (Nakamura and Fujiki, 1968). This agrees well with the molecular weight determined by light scattering of the reduced enzyme in 6 M Gdn·HCl.

Sedimentation velocity measurements were also made at 5° on glucose oxidase in 6 M Gdn·HCl and in 6 M Gdn·HCl–0.1 M βME. Extrapolating these data to $c = 0$ gave sedimentation coefficients of 1.08 and 0.75 S for glucose oxidase in 6 M Gdn·HCl and 6 M Gdn·HCl–0.1 M βME respectively. The s^0 values corrected to 25° are approximately 1.71 and 1.19 S in these two solvents. Since the theoretical (Flory, 1953; Tanford, 1961) dependence of the sedimentation coefficient varies approximately with the square root of the molecular weight, we can calculate a ratio of molecular weights, as shown in eq 7, if we assume the enzyme is in a randomly

$$s^0_{\text{Gdn·HCl}}/s^0_{\text{Gdn·HCl–}\beta\text{ME}} = (M_{\text{Gdn·HCl}}/M_{\text{Gdn·HCl–}\beta\text{ME}})^{0.5-X} \quad (7)$$

coiled conformation in both solvents. The exponent in eq 7 is 0.5 in a poor solvent and 0.45 in a thermodynamically good solvent. Tanford *et al.* (1967) have found an exponent of 0.473 best fits their data for proteins in 6 M Gdn·HCl–0.1 M βME. Using 0.473 as the exponent, eq 7 yields a molecular weight ratio of 2.15. This is in reasonable agreement with the molecular weight ratio 2.0 determined directly by light scattering and it lends support to our earlier conclusion that glucose oxidase is a subunit enzyme containing two covalently linked polypeptide chains.

The sedimentation coefficient data can also be used to estimate the absolute molecular weight of the reduced and denatured enzyme. The relationship, $s^0/(1 - \nu\rho) = 0.286n^{0.476}$, has been determined (Tanford *et al.*, (1967)) experimentally to hold for a variety of proteins in 6 M Gdn·HCl–0.1 M βME. Here, n is the number of amino acid residues in the protein and the other terms have their usual significance. For reduced glucose oxidase, using a value of 0.704 for the partial specific volume in 6 M Gdn·HCl–0.1 M βME (approximately 0.01 less than in buffer), a value of $n = 646$ residues is calculated. Assuming for this glycoprotein that n is the total number of residues (amino acids + carbohydrates) and that the average residue molecular weight is 120 (110 average for amino acids and 180 average for the carbohydrates comprising 16% of the protein), a molecular weight of 77,400 is calculated. This is very near the molecular weight determined by light scattering and is slightly lower than half the molecular weight of the active enzyme determined by sedimentation velocity in buffer solution.

Discussion

Light-scattering and sedimentation velocity results reported herein indicate that glucose oxidase isolated from *A. niger* is a subunit enzyme composed of two, covalently linked, polypeptide chains of approximately equal molecular weight. The average molecular weight of the constituent polypeptide chains was measured in 6 M Gdn·HCl–0.1 M βME and found to be 80,000, whereas the unreduced (intact) enzyme in 6 M Gdn·HCl was found to have a molecular weight of 160,000. The latter is essentially the same as that found for the native enzyme in buffer. The effect of the reducing agent, β-mercaptoethanol, on the molecular weight of the denatured enzyme provides strong evidence for the two polypeptide subunits being covalently linked through disulfide bonds. The exact number of intermolecular disulfide bonds is not known but our amperometric titration data indicate that a maximum number of two disulfides are involved. These same titration data also indicate that the two polypeptide chains comprising glucose oxidase are not identical because an odd number (one) of sulfhydryl groups were titrated. However, in view of the very small content of disulfides and sulfhydryls in the enzyme, we feel that additional supporting evidence is needed to firmly establish this result.

Viscometry was used extensively to probe the subunit structure of glucose oxidase. One aspect of the viscosity data which deserves further comment is the effect of reducing agent concentration. The data indicate that the intrinsic viscosity of glucose oxidase denatured in 6 M Gdn·HCl is independent of reducing agent concentration between 0 and 0.5 M βME. Other reducing agents such as sodium sulfite and dithiothreitol also have no effect on the intrinsic viscosity. Ordinarily this would indicate that the reducing agent does not influence or change the molecular weight of the enzyme. However, in the present study such an interpretation is not valid because light-scattering and sedimentation velocity measurements definitely show a twofold decrease in the molecular weight of the enzyme upon addition of only 0.1 M βME. Evidently the one or two disulfides per 160,000 molecular weight constitute such a low concentration of cross-links that they do not substantially affect the conformation of the enzyme in solution. A model of glucose oxidase with its two subunit chains cross-linked at or near their termini would be consistent with the viscosity data.

A second interesting aspect of the viscosity data is the absolute magnitude of the measured intrinsic viscosity. Tanford *et al.* (1967) have shown that proteins denatured and reduced in 6 M Gdn·HCl–0.1 M βME are in a random-coil conformation and that their intrinsic viscosity can be correlated with their molecular weight. A number of proteins obey the relationship shown in eq 8, where $[\eta]$ is the intrinsic viscosity and n is the number of amino acid residues in the protein.

$$[\eta] = 0.716n^{0.66} \quad (8)$$

For glucose oxidase with a molecular weight of approximately 160,000, assuming a single-chain enzyme and taking into account the 16% carbohydrate, we calculate an intrinsic viscosity of approximately 80 ml/g. When compared to the experimentally determined value of 30 ml/g in 6 M Gdn·HCl–0.1 M βME, we see that the single-chain model does not even approximate the experimental value. Assuming now that the enzyme is dissociated into two polypeptide chains having molecular weights of 80,000, as is indicated by our light-scattering measurements, the calculated viscosity is in the

range of 50 ml/g. This calculated value is also well above the observed viscosity of 30 ml/g. It appears, therefore, that eq 8 overestimates the intrinsic viscosity of glucose oxidase in 6 M Gdn·HCl–0.1 M βME. This is not too surprising in view of the fact that glucose oxidase is a branched glycoprotein containing 16% carbohydrate and eq 8 is an empirical correlation for linear proteins.

Finally, it is appropriate to consider here the recent work of Yoshimura and Isemura (1971) on glucose oxidase isolated from *Penicillium amagasakiense*. From sedimentation velocity and equilibrium studies, these authors concluded that the glucose oxidase molecule consists of four polypeptide chains equal in molecular size (45,000) and that two polypeptide chains are held together by a disulfide bond to form a dimer (81,000) and two dimeric units associate noncovalently to form a tetramer (160,000). The dimer species are formed by denaturing glucose oxidase in 6 M Gdn·HCl and the monomeric species are formed by reducing the dimers with 0.3 M βME. These results are significantly different from those found in the present study and a dimer model in which glucose oxidase from *A. niger* is composed of two polypeptide chains equal in molecular size and covalently linked by disulfide bonds is consistent with our data. Other workers have previously noted significant differences in these two enzymes and it now appears that subunit structure is yet another property that can be added to the long list of physical properties distinguishing (Swoboda and Massey, 1965; Bodmann and Walter, 1965; Nakamura and Fujiki, 1968) the glucose oxidase enzymes isolated from *A. niger* and *P. amagasakiense*.

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